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Original Article

Time of day determines Arabidopsis transcriptome and growth dynamics under mild drought

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ABSTRACT

Drought stress is a major problem for agriculture worldwide, causing significant yield losses. Plants have developed highly flexible mechanisms to deal with drought, including organ- and developmental stage-specific responses. In young leaves, growth is repressed as an active mechanism to save water and energy, increasing the chances of survival but decreasing yield. Despite its importance, the molecular basis for this growth inhibition is largely unknown. Here, we present a novel approach to explore early molecular mechanisms controlling Arabidopsis leaf growth inhibition following mild drought. We found that growth and transcriptome responses to drought are highly dynamic. Growth was only repressed by drought during the day, and our evidence suggests that this may be due to gating by the circadian clock. Similarly, time of day strongly affected the extent, specificity, and in certain cases even direction of drought-induced changes in gene expression. These findings underscore the importance of taking into account diurnal patterns to understand stress responses, as only a small core of drought-responsive genes are affected by drought at all times of the day. Finally, we leveraged our high-resolution data to demonstrate that phenotypic and transcriptome responses can be matched to identify putative novel regulators of growth under mild drought.

Key-words: leaf growth regulation; mild drought response; time-course transcriptomics.

INTRODUCTION

Drought stress is a huge environmental problem, causing tremendous agricultural yield losses (Araus *et al.* 2002; Boyer 1982). Around 40% of global land area is situated in arid or semiarid climates (Fedoroff *et al.* 2010; Marris 2008) and this problem will most likely worsen in the next decades because of rising temperatures, increasing the duration of drought periods (Fedoroff 2010). Drought can occur in multiple levels of severity and can hit during all stages of plant development, requiring specific responses (Bray 2004; Claeys & Inzé 2013; Langridge & Reynolds 2015; Verslues *et al.* 2006). When drought occurs during vegetative growth, plants react in a flexible way and reprogram growth (for reviews, see Claeys & Inzé 2013; Pierik & Testerink 2014). Repression of leaf growth is among the first responses to drought, and because this is one of the factors at the origin of the yield losses caused by drought (Correa-Tedesco *et al.* 2010), efforts have been made to understand and eventually circumvent or delay this growth inhibition.

Leaf growth is mediated by two tightly spatio-temporally regulated cellular processes: cell division and cell expansion. In *Arabidopsis thaliana*, growth of emerging leaves is first driven by cell proliferation, generating the pool of cells that subsequently enter cell expansion to drive the so-called expansive leaf growth (Andriankaja *et al.* 2012; Donnelly *et al.* 1999). Drought was found to negatively affect both cell proliferation and expansion in different natural variants of Arabidopsis and maize (Baerenfaller *et al.* 2012; Bonhomme *et al.* 2012; Clauw *et al.* 2015; Harb *et al.* 2010). Although constraints in leaf hydraulics negatively affect leaf expansion under severe or prolonged drought (Caldeira *et al.* 2014; Pantin *et al.* 2013; Tardieu *et al.* 2014), evidence suggests that drought also inhibits leaf growth even when leaf hydraulics are maintained (Bonhomme *et al.* 2012; Parent *et al.* 2010; Tang & Boyer 2002), pointing towards active signaling in leaf growth regulation under drought. Moreover, molecular cascades involving crosstalk between ethylene and DELLA proteins have been shown to be rapidly induced to shut down leaf growth under mild, *in vitro*-applied osmotic stress (Claeys *et al.* 2012; Dubois *et al.* 2013; Skirycz *et al.* 2011a). These ethylene and DELLA-mediated mechanisms were not observed upon exposure to in soil-applied drought stress (Baerenfaller *et al.* 2012; Clauw *et al.* 2015). However, these recent studies focused on long-term drought responses (Baerenfaller *et al.* 2012; Clauw *et al.* 2015; Des Marais *et al.* 2012; Harb *et al.* 2010; Wilkins *et al.* 2010), leaving the early molecular responses of actively growing leaves of plants exposed to mild drought stress unexplored.

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Author contributions: M. D. and H. C. designed research, generated plant material, performed research and analyzed data; L. V. d. B. generated plant material; M. D., H. C. and D. I. wrote the paper.
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In Arabidopsis, the relative growth rate (RGR; generated area per unit of existing area per unit of time) of leaves varies according to the developmental stage of the leaf as well as to the time of day: young leaves have higher growth rates during the day, while older leaves grow more during the night (Pantin *et al.* 2011; Pantin *et al.* 2012; Ruts *et al.* 2012; Schurr *et al.* 2006; Wiese *et al.* 2007). In dicot species, these diurnal growth rhythms are generally not affected by environmental factors that are linked with day/night rhythms, such as light and temperature (Caldeira *et al.* 2014; Poiré *et al.* 2010). In contrast, diurnal rhythms are disturbed in circadian clock mutants and mutants affected in starch metabolism, indicating that leaf growth is endogenously controlled by a mechanism integrating metabolic signals and the circadian clock (Nozue & Maloof 2006; Poiré *et al.* 2010; Ruts *et al.* 2012). In a simplified view, the core circadian clock machinery is based on transcription-translation feedback loops between two major components: the LHY/CCA1 (late elongated hypocotyl and circadian clock associated 1) complex and TOC1 (timing of cab expression 1). The LHY/CCA1 complex, highly expressed in the morning, represses the expression of *TOC1*, which itself encodes a repressor of *LHY* and *CCA1*. As a result, oscillating expression patterns of *LHY*/*CCA1* and *TOC1* trigger the expression of morning and evening genes, respectively (reviewed in Hsu & Harmer 2014). Several studies focused on the molecular connection between the circadian clock and hypocotyl growth, but little is known about the molecular players linking the clock to leaf growth (Arana *et al.* 2011; Filo *et al.* 2015; Ruts *et al.* 2012) and about how they are influenced by drought stress.

Here, we present a novel approach to explore the short-term molecular mechanisms underlying leaf growth inhibition following drought by taking into account the diurnal growth rhythms. Using the Weighing, Imaging and Watering Automated Machine (WIWAM) to precisely control soil water content, we exposed young Arabidopsis seedlings to mild drought stress and tracked the growth and transcriptional responses over time specifically in actively growing Arabidopsis leaves.

MATERIALS AND METHODS

Plant lines

lhy (N531092) and *cca1* (N513233) mutant lines were obtained from the NASC collection. The *toc1-101* mutant was a kind gift from Dr Yanovsky (Instituto Leloir, Buenos Aires, Argentina). FLAG_314D04 (*erf2*) and FLAG_157D10 (*erf8*) mutants were obtained from the ATRC (IJBP, Versailles, France) collection. All mutants were upscaled and grown with the respective wild type.

Plant growth conditions

All reported experiments were performed on the WIWAM platform (http://www.wiwam.be). In our setup, four seedlings were grown per pot in order to grow 864 seedlings simultaneously on the platform. The seeds were sown in 85 g +/−1 g of Saniflor compost (Van Isreal N.V., Geraardsbergen, Belgium) with an absolute water content of on average 70%. The seeds were covered with plastic foil until 5 d after stratification (DAS), when the automated watering started. When mutants were analyzed, the pots were randomized to homogenously mix mutant and wild-type plants. Plants were grown under a long-day regime (light from 6 AM until 10 PM, intensity 110–120 μmol $m^{-2}s^{-1}$) at 21 °C. All plants were watered daily from 5 days after stratification (DAS) until 11 DAS with a well-watered (WW) regime of soil relative water content (RWC) 69% (2.2 g_{water}/g_{soil}). At 12 DAS, half of the pots (random positions) were maintained at the WW regime while the other half were not watered until the end of the experiment at 17 DAS. At this final time point, soil RWC reached 55% (1.2 g_{water}/g_{soil}).

Leaf area and cellular measurements

All described measurements were performed on the third true leaf of the rosette. Destructive leaf area measurements were performed by cutting the leaf, clearing it in 100% ethanol and mounting it on microscopic slides in lactic acid. Leaves were photographed with a microscope and the area was measured based on the pictures using ImageJ v1.45 (NIH; https://rsb.info.nih.gov/ij/). Harvesting was performed at 6 AM and at 10 PM. Leaf area measurements were performed in four biological repeats. For the growth experiments performed on the same leaf over time, as represented in Fig. 1d and 1e, an imprint of the abaxial surface of the leaf was taken with dental resin (Kagan *et al.* 1992) every morning and evening. The imprints on the resin were subsequently photographed and measured with ImageJ. Five to eight leaves were analyzed per condition per biological repeat. For subsequent cellular analysis, the leaf imprints were overlaid with a thin layer of nail polish. The nail polish copy of the imprint was analyzed by scanning electron microscopy. A region of approximately 200 cells was followed over time, and the number of cells that divided within that region between two consecutive time points (as shown in Fig. S1E) was counted. The expansion of the selected zone of cells was calculated using ImageJ. The absolute expansion rate of the zone was divided by the number of cells to estimate the cell expansion rate. For the leaf area measurements to compare mutant and wild-type phenotypes, as shown in Figs 3 and 4, the third true leaf of 30–50 plants was harvested only at the end of the experiment, at 17 DAS, and measured as described previously.

Sampling for expression analysis and qRT-PCR

All described experiments were performed on the third true leaf in three biological replicates. Per treatment and per time point, four leaves were harvested per replicate at 4 AM, 8 AM, 12 PM, 4 PM, 8 PM, 12 AM on 12 DAS (starting from 8 AM), 13 DAS, 14 DAS, 15 DAS and 16 DAS (until 8 AM). The leaves were pooled and flash frozen in liquid nitrogen immediately upon harvest. For harvesting during the night, a low-intensity green light was used. RNA extraction, cDNA

synthesis and qRT-PCR were performed as previously reported (Dubois *et al*. 2013). cDNA was synthesized from 200–500 ng RNA. Primers were designed with the QuantPrime website.

RNA sequencing and differential expression analysis

The sequencing was performed at the Nucleomics Core Facility (VIB, Leuven, Belgium, www.nucleomics.be). Library preparation was performed with the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Quality was checked with the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA), and clusters were generated through amplification using the TruSeq SE Cluster Kit (Illumina, San Diego, CA, USA). Samples were sequenced on a HiSeq 2000 in single-end mode with reads of 50 bp in length. Subsequent data analysis steps were performed in Galaxy. The quality of the sequences was verified with FASTQC (http://bioinformatics.babraham.ac.uk/projects/fastqc/), and filtering of the adaptor and other overrepresented sequences was performed with the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). The remaining reads were mapped to the Arabidopsis reference genome, using GSNAP, according to TAIR10 (TAIR10_chr_all.fas; ftp.arabidopsis.org). Reads that did not map to a unique position were removed using SAMtools (v0.1.18). Differential expression analysis was performed with multifactorial ANOVA using the EdgeR and ggplot2 libraries in R3.0.1 (https://www.r-project.org). Rough counts were normalized to the library size. Very lowly expressed genes were removed by filtering for genes with counts >5 cpm (counts per million) in at least three samples. The new libraries were normalized by TMM. A generalized linear model was applied with time and treatment as factors using the glmFit function. Next, significant interactions were extracted using the glmLRT function and the interaction term as a coefficient. Differentially expressed genes in drought stress versus WW conditions at each time point were calculated using predefined contrasts for each group. The cut-off was set on FDR = 0.05 and $\log_2$(fold change) > 0.2. To calculate the effects of drought stress on the amplitude of oscillation of expression, we defined the amplitude as the difference in expression level between the highest and the lowest observed expression within a treatment. To reduce noise, only highly expressed genes (>5 cpm at each time point) with clear changes in expression throughout the day (increase in transcript levels by more than 25% of the lowest expression level) were considered. We then compared this amplitude under WW conditions with that under drought and considered that the amplitude was affected by drought when both differed more than 1.5-fold. Clustering was performed in TMEV (www.tm4.org) using K-means clustering with 50 clusters and 200 iterations. The clusters were curated manually to remove the genes that were wrongly assigned to the clusters. Gene ontology enrichment analysis was performed using the PLAZA Workbench (http://bioinformatics.psb.ugent.be/plaza). All sequencing data is available on ArrayExpress.

RESULTS

Drought inhibits leaf growth within 3 days following stress onset, only during the day

To explore the dynamics of leaf growth under drought, we developed a mild drought stress assay enabling to track the growth of young Arabidopsis leaves over time. Seedlings were grown on an automated watering platform (WIWAM (http://www.wiwam.be), Fig. S1A and S1B; Skirycz *et al.* 2011b) and water was first withheld at 12 days after stratification (DAS; Fig. 1a), when the actively growing third true leaf, used as a model organ for all presented experiments, is 1 mm in length (growth stage 1.03; Fig. S1C). At this stage, the leaf is composed of both proliferating and expanding cells. Progressive drying of the soil was maintained for 5 days, and leaf area was accurately measured by harvesting the third leaf from 20 different plants every morning and evening during the progressive drought period. On the final harvesting time point, 5 days since last watering (DSLW), the soil humidity of the mild drought pots had dropped from 2.2 (soil relative water content (RWC) 69%) to 1.2 g_{water}/g_{soil} (soil RWC 55%), resulting in a final leaf area reduction of on average 20% (Fig. 1b).

Because leaf growth rates are known to be different during day and night (Dornbusch *et al*. 2014; Nozue & Maloof 2006), relative growth rates (RGR) were calculated separately to quantify growth during the day and during the night (dRGR and nRGR, respectively). In well-watered (WW) conditions, dRGR was higher than nRGR and gradually decreased over time (Fig. 1c). During drought stress, the decrease in dRGR was more pronounced than that under WW conditions ($P = 0.06$; *t*-test of the slopes), reaching nRGR levels much faster. Remarkably, nRGR was completely unaffected by drought ($P = 0.51$; *t*-test of the slopes).

Leaf growth was visibly affected by drought from the fourth DSLW onwards (Fig. 1b). However, we suspected that subtle growth-inhibitory effects might be masked when using the average of a pool of 20 leaves, and we therefore followed the growth of individual third leaves over time by taking non-destructive leaf imprints every morning and evening during the progressive drought period (Fig. S1D). This method revealed drought-induced growth inhibition already after three DSLW (Fig. 1d). At the cellular level, this growth inhibition observed during the third day following stress onset resulted from a decrease in both cell division (−48%; $P = 0.001$) and cell expansion (−31%; $P = 0.06$) (Fig. 1e & Fig. S1E). Together, these results show that young leaves of plants exposed to stress reduce their growth during the day only, from the third DSLW onwards, through inhibition of both cell division and cell expansion.

Time of day determines the extent and the specificity of the drought response

To understand the short-term molecular mechanisms coordinating growth responses under mild drought stress by taking into account the effect of the time of day, we profiled the transcriptome of third leaves exposed to drought and to WW conditions around the moment of leaf growth inhibition, during

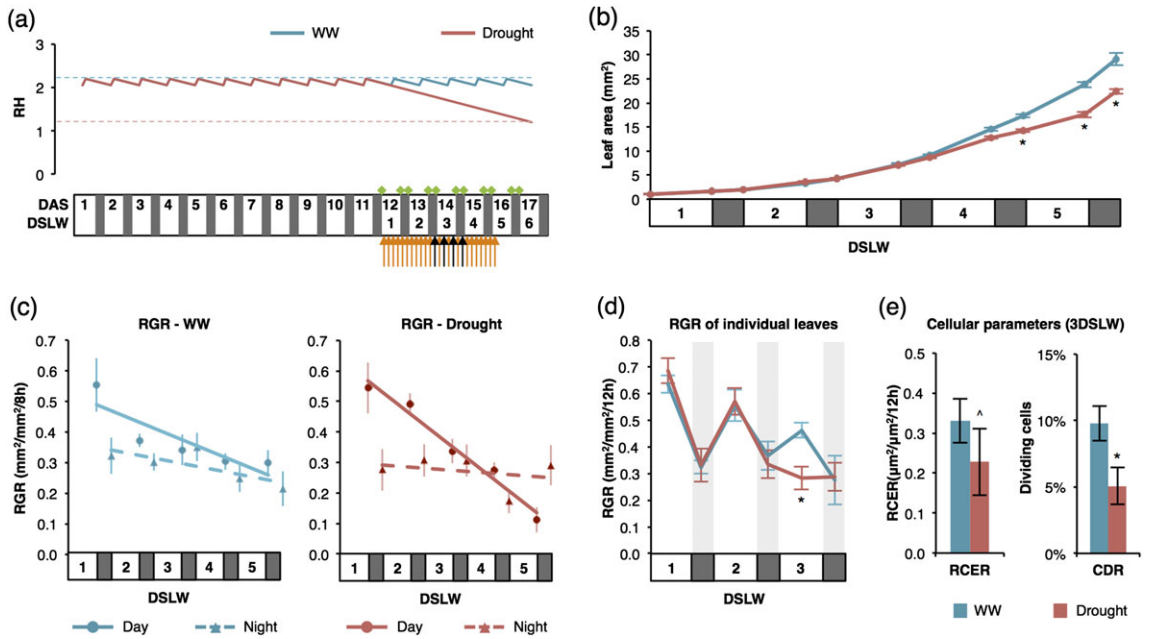

Figure 1. Experimental setup and leaf growth dynamics under well-watered and drought stress conditions. (a) Arabidopsis plants were grown under well-watered (WW, blue) conditions ($2.2 g_{water}/g_{soil}$) until 12 days after stratification (DAS). Subsequently, half of the pots were exposed to a mild drought stress treatment (which reached $1.2 g_{water}/g_{soil}$ at the end of the time course, red), while the other pots were kept under the WW regime. Harvests were performed from before the stress onset (at 12 DAS) until 17 DAS, twice a day (morning and evening) for the leaf growth measurements (green diamonds), and every 4 h for expression analyses (orange arrows). Samples used for RNA sequencing are indicated with black arrows. RH = relative humidity of the soil (g_{water}/g_{soil}), DSLW = days since last watering. (b) Average leaf area over time of the third leaf ($n = 20$) under WW and drought stress conditions. (c) Average relative growth rate (RGR) of the third leaf ($n = 20$) under WW and drought stress conditions during the day and during the night. (d) Average RGR of the third leaf ($n = 7$) followed using leaf imprints. (e) Cellular measurements of the third leaf ($n = 7$) during the third DSLW. RCER = relative cell expansion rate, CDR = cell division rate. Four biological repeats were performed ($n = 20$ per repeat) for b and c, and two for d and e ($n = 7$ per repeat). Error bars represent standard errors in all panels. * $P < 0.05$, ^ $P < 0.1$.

day and night: at 4 AM, 12 PM, 8 PM on the third DSLW and at 4 AM on the fourth DSLW (labeled hereafter as 4 AM') (Fig. 1a).

Although the drought stress was still mild at the investigated time points, the expression of 5659 genes was significantly affected in at least one time point (Dataset S1). Notably, more than half of the differentially expressed (DE) genes (3016; 53%) had not been previously identified in comparable datasets derived from shoot tissue of plants exposed to mild or moderate drought (Dataset S1 and Table S1A) (Baerenfaller *et al.* 2012; Clauw *et al.* 2015; Des Marais *et al.* 2012; Harb *et al.* 2010; Wilkins *et al.* 2010). Strikingly, the extent of the drought response clearly depended on the time of day, as shown by the amount of DE genes at each time point (Fig. 2a & Fig. S2A). The effect of progressive drought was clear from the increase in DE genes between the two comparable night time points (4 AM and 4 AM'). However, the number of DE genes varied throughout the day, reaching a low point at noon, followed by a peak at 8 PM, after which it decreased again. Drought-induced transcriptome changes thus do not gradually increase with the stress level throughout a day, but the time of day determines the extent of the drought response.

Direct comparison of the DE genes between the time points showed that the large majority was DE at only one (78%) or two (17%) time points (Fig. 2b). Surprisingly, only 29 genes (0.5%) were DE in the same direction along the whole time course (Table S1B). A gene ontology search on these genes showed that they encode proteins involved in classical drought-responsive processes such as cell wall loosening, proline accumulation, lipid and wax biosynthesis, and abscisic acid (ABA) signaling. To further validate these observations, we performed a high-resolution expression analysis, harvesting third leaf samples every 4 h during the five DSLW (Fig. 1a). In this detailed time-course analysis, the expression of the proline dehydrogenase *ERD5*/*ProDH1* showed consistent down-regulation by drought from the first time point onwards, despite strong oscillation of gene expression throughout the day, indicating that it is a very responsive drought marker (Fig. 2c). Similarly, expression of the ABA receptor *PYL6* was robustly down-regulated from 28 h after water restriction onwards (Fig. 2c). We further compared above gene expression data with the five other comparable datasets (Table S1B). Out of 29 of these common drought-responsive genes, 26 (90%) responded similarly to drought in the other published datasets, again illustrating that they are classical drought-responsive genes. As these studies were conducted on leaves at different developmental stages ranging from proliferation to maturity, this suggests that these common drought genes are most likely involved in general drought-responsive processes rather than in growth regulation. This is further supported by the

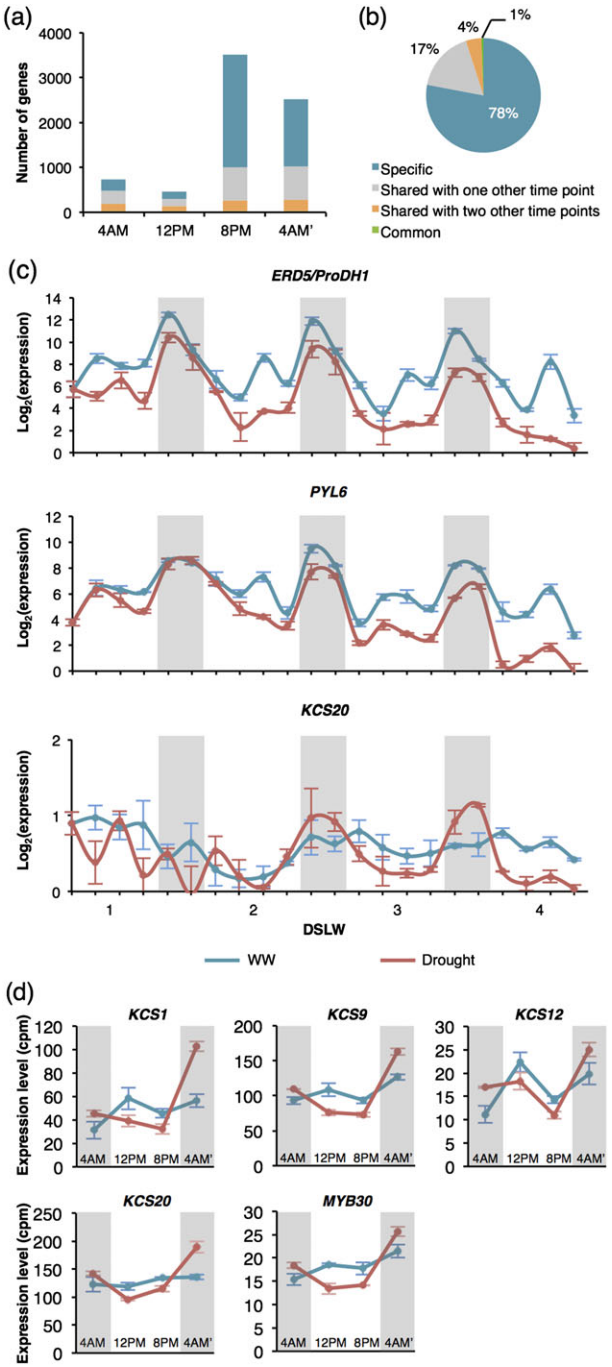

Figure 2. Gene expression analysis following mild drought stress. (a) Number of differentially expressed genes with FDR < 0.05 at 4 AM, 12 PM, 8 PM during the third day since last watering (DSLW) and at 4 AM during the fourth DSLW (labeled 4 AM'). (b) Comparison of the differentially expressed genes between the time points. (c) Expression levels of *ERD5*/*ProDH1*, *PYL6* and *KCS20* during 4 days following drought onset. (d) Expression levels of the *KCS* genes and their regulator *MYB30* in the transcriptomics data. Error bars represent standard errors for three biological replicates. WW = well-watered, cpm = counts per million.

observation that these genes were DE at each time point, while drought stress clearly does not affect leaf growth equally throughout day and night.

Time of day affects the direction of the drought response

As illustrated previously for *ERD5*/*ProDH1* and *PYL6*, expression levels under WW conditions clearly oscillated throughout the day (Fig. 2c). In total, the expression of 62% of the 18 750 genes expressed in developing leaves was influenced by the time of day under WW conditions (FDR < 0.05), and 6394 genes clearly oscillated with expression changes larger than 25% throughout the day. To explore the general effect of drought on these time-dependent expression patterns, we calculated drought-induced changes in amplitude of oscillations. While 22% of the transcript oscillations showed a clear (>1.5-fold) reduction in amplitude under drought, 11% of the genes showed an increased amplitude (Fig. S2B). Additionally, 505 genes of which transcript levels did not clearly oscillate under WW conditions showed expression changes larger than 25% throughout the day under mild drought conditions.

Intrinsically, drought-induced amplitude changes are expected to result from opposite effects of drought at different times of the day. In our dataset, 166 genes were significantly affected by drought in the opposite direction during day and night, and these could be classified into four clusters (Fig. S3A). Strikingly, the cluster of 49 genes repressed by drought during the day but up-regulated by drought during the night is strongly enriched for genes encoding enzymes for very long chain fatty acid (VLCFA) elongation, such as *KCS1*, *KCS9*, *KCS12*, *KCS20* and their regulator *MYB30* (Fig. 2d). We validated this for *KCS20* by qRT-PCR along the complete time course (Fig. 2c). Surprisingly, another example of how the time of day affects the direction of drought-induced gene expression changes was the classical drought-induced gene *DREB2A* (Sakuma *et al.* 2006), whose expression was from the third DSLW onwards induced by drought during the day but repressed by drought at night (Fig. S3B).

The circadian clock affects the drought response and *vice versa*

To further explore whether the changes in diurnal expression patterns result from altered circadian clock regulation under drought, we measured the expression of central circadian clock genes along the complete time course. Drought stress did not drastically affect the expression of *TOC1*, *LHY* and *CCA1* (Fig. S4). However, subtle but statistically significant effects could be consistently observed for both *TOC1* and *LHY*, which under drought conditions reached lower minimal expression levels during their oscillations (in the morning and evening, respectively) (Fig. S4).

Next, we exposed loss-of-function lines for each of these clock components to mild drought stress. When comparing the relative leaf area reduction caused by drought stress, the *cca1* and *lhy* mutants were affected by drought to the same extent as wild-type plants, but the *cca1* mutant was smaller under WW conditions (Fig. 3). In contrast, the *toc1* mutant was more sensitive to drought, as evidenced by a leaf area reduction of on average 32.2% under drought, as compared with 20.8% in the wild type ($P = 0.007$; ANOVA, Genotype x Treatment

interaction) (Fig. 3b). To obtain more insight into this hypersensitive phenotype, the growth of individual *toc1* leaves was followed over time using leaf imprints. In addition to being affected by drought to the same extent as wild-type plants during the day, *toc1* leaf growth was also reduced by drought stress during the night, when wild-type leaf growth was unaffected (Fig. S5). Thus, when the *TOC1* gene is mutated, the drought-induced growth inhibition not only occurs during the day period but also at night, resulting at the end of the experiment in the drought-hypersensitive phenotype of *toc1* seedlings.

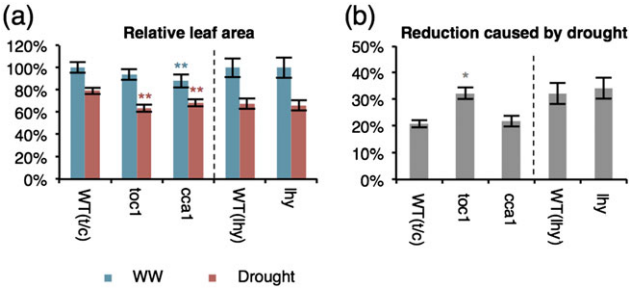

Figure 3. Analysis of core circadian clock mutants under drought. (a) Average area of the third leaf of the circadian clock mutants under well-watered (WW) or drought conditions measured at six days since last watering (DSLW), relative to the respective wild type under WW conditions. ** $P < 0.001$ (ANOVA; TukeyHSD), compared with the respective wild type under the same condition. (b) Relative reduction in average leaf area caused by drought in each line at six DSLW. * $P < 0.05$ (ANOVA; Treatment x Genotype interaction), compared with the respective wild type. For both panels, error bars represent standard errors of three biological replicates.

Matching growth and transcript dynamics to identify novel regulators

To identify growth-related mechanisms under drought, genes that were DE in at least one of the four selected time points were clustered. Clusters with profiles correlating or anti-correlating with growth dynamics (e.g. high expression during the day but low expression at night) were selected. Interestingly, these clusters, comprising 228 genes (Dataset S2), were enriched for distinct ontology terms compared with the full dataset, such as GO classes related to the phytohormones ethylene, jasmonic acid (JA), and gibberellins (GAs). Because previous *in vitro* experiments conducted on mild osmotic stress have shown that the growth-inhibitory stress response is orchestrated by transcription factors (Claeys *et al.* 2012; Dubois *et al.* 2013), we selected the transcription factors from this list for further analysis. For most tested genes, the expression pattern was confirmed in two additional biological repeats (Fig. S6). As a proof-of-concept, we measured growth of loss-of-function lines for six genes from different transcription factor families (ERF, WRKY, MYC/bHLH; Fig. 4 and Fig. S7) under mild drought. Interestingly, two of the six tested lines had a significantly altered growth response to mild drought: *erf2* and *erf8* (Fig. 4). *erf2* mutants were indistinguishable from wild type under WW conditions but were significantly more sensitive to stress ($P < 0.001$; ANOVA, Genotype x Treatment interaction) (Fig. 4a and b). *ERF8*, in contrast, negatively affected leaf growth already under control conditions, because *erf8* mutants were 27% larger than wild type ($P = 2.6E\text{-}6$; ANOVA, TukeyHSD) (Fig. 4a). Under drought, *erf8* mutants had leaves that were 20% larger than controls ($P = 2E\text{-}16$; ANOVA, TukeyHSD; Fig. 4a) but were more affected by drought ($P = 0.037$; ANOVA, Genotype x Treatment interaction; Fig. 4b). This data thus shows that by combining the dynamics of a phenotype with the dynamics of gene expression, promising candidate genes regulating the phenotype of interest can be identified.

DISCUSSION

Plant responses to drought are extremely complex, largely depending not only on the severity and duration of the stress but also on the organ and its developmental stage. Here, we chose the growing Arabidopsis as a model to study organ growth regulation in response to drought. The chosen model leaf was still actively growing, enabling the study of growth inhibition, but already sufficiently large to be easily and efficiently harvested in a high-resolution time-course setup. In Arabidopsis, it is very challenging to track the growth dynamics of small, actively growing leaves of plants exposed to stress. Generally, leaf growth is approximated using top-view imaging, which is perturbed by the diel leaf movements (Harb *et al.* 2010; Clauw *et al.* 2015; Skirycz *et al.* 2011b; Granier *et al.* 2006; Tisné *et al.* 2013; Apelt *et al.* 2015 and reviewed in Vanhaeren *et al.* 2015), or obtained from immobilized growing leaves (Wiese *et al.* 2007). Whereas measurements at the rosette level have shown that mild drought triggers growth inhibition from 10 days following drought onset onwards (Clauw *et al.* 2015), our method showed that the growth rate of Arabidopsis leaves slows down significantly already 3 days following the watering arrest, when soil RWC had dropped by just 6% (from 69 to 63%). This highlights the sensitivity of leaf growth to changes in water status. Growth is repressed specifically during the day, when young leaves grow the fastest under well-watered conditions, which is likely an active mechanism of the plant to save resources when they are most scarce.

Non-destructive accurate measurements are often performed in crop species (Matt *et al.* 1998; Poiré *et al.* 2010; Tardieu & Granier 2000; Tardieu *et al.* 2014), particularly in maize, where time-course leaf growth measurements following drought have shown much faster growth-inhibitory responses within hours upon water withholding (Caldeira *et al.* 2014). Although we cannot fully exclude that technical limitations of our setup explain part of this important difference in timing, this fits with the observations that growth of maize leaves appears to be less controlled by the circadian clock (Poiré *et al.* 2010) but is instead very dependent on hydraulics (Caldeira *et al.* 2014). Maize leaves may therefore react much earlier to water deficits than those of Arabidopsis for which growth in changing environmental conditions is known to be mainly controlled by clock-regulated mechanisms. We demonstrated with the *toc1* mutant that disrupting part of the core circadian clock increases the negative effect of drought on leaf growth. Importantly, only

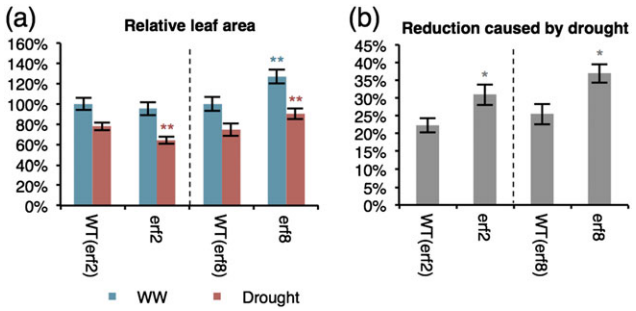

Figure 4. Leaf area measurements of *erf2* and *erf8* mutants exposed to drought. (a) Average area of the third leaf of *erf2* and *erf8* mutants under well-watered (WW) and drought conditions measured after six days since last watering (DSLW), relative to the respective wild type under WW conditions. ** $P < 0.001$ (ANOVA; TukeyHSD), compared with the wild type under the same condition. (b) Relative reduction in average leaf area caused by drought in each line at six DSLW. * $P < 0.05$ (ANOVA; Treatment x Genotype interaction), compared with the respective wild type. For all panels, error bars represent standard errors of three biological replicates.

growth under drought is affected, while no growth defect was observed under WW conditions, suggesting a specific effect on the drought response rather than a general loss of fitness of *toc1*. Recently, loss-of-function of TOC1 was shown to trigger hypersensitivity to biotic stress as well (Zhou *et al.* 2015). Both under biotic stress and under drought, *TOC1* expression was down-regulated, pointing towards similar stress response mechanisms. This down-regulation was previously shown to reinforce the circadian clock under stress. Under biotic stress, the defense response is known to be gated by the circadian clock, enabling expression of defense genes during the day but restricting it during the night, saving resources for growth (Baldwin 2013; Wang *et al.* 2011; Zhang *et al.* 2013). A similar mechanism could function under mild drought conditions, where growth is also preserved during the night, while it is shut down during the day, when other defense responses might be activated. We could speculate that *DREB2A* could be involved in a similar growth-defense balance under drought conditions, because it is induced by drought during the day but repressed by drought at night. *DREB2A* is a defense gene of which the expression is controlled by the growth-regulating factor7 (GRF7) (Kim *et al.* 2012). It thus would make biological sense that *DREB2A* is up-regulated during the day when the 'defense' response to drought is on, but that it would need to be down-regulated by drought at night to allow growth to proceed.

At this mild level of stress at which growth was affected (6% decrease in soil RWC), drought also affected the expression of thousands of genes. While there is no absolute relationship between transcriptome and proteome changes, and care should thus be taken when interpreting transcriptomics data, for growing organs it is nearly impossible to obtain enough tissue to reliably quantify levels of growth regulators at the proteome level, and we therefore studied the transcriptome as a proxy for proteome changes. While many studies have performed transcriptomics after week(s) of drought, studying expression changes at earlier time points following drought holds huge potential to uncover a new set of relatively rapid drought responses orchestrating the now already well-characterized later stress responses. We refer to the responses described in this study as short-term responses to contrast them with the existing drought response literature. We have also shown that over the course of one day of progressive soil drying, the extent of the drought response on the transcriptome level did not increase gradually. Clearly, the time of day determined the extent of the drought response, as well as the identity of the genes induced at that specific time point. Even more surprisingly, examples of genes that were regulated in different directions depending on the time of day were found, such as the genes encoding enzymes contributing to the subsequent steps of VLCFA biosynthesis, that is *KCS20*, *KCS9* and *KCS1* (Kim *et al.* 2013; Lee *et al.* 2009; Todd *et al.* 1999), which are down-regulated by drought during the day but up-regulated by drought during the night. It is possible that VLCFAs, which are building blocks for cuticular wax, are mostly synthesized during the night to thicken the cuticula at night and prevent extensive evaporation from dawn onwards (Seo & Park 2011). Additionally, recent studies uncovered emerging roles for VLCFAs as signaling molecules, synthesized in the leaf epidermis but transported to inner cell layers to control cellular division and growth (Nobusawa *et al.* 2013). Although the latter process is still poorly understood, we do not exclude that this new role of VLCFAs might be important for leaf growth regulation under mild drought. Overall, we showed that transcriptomics data at only one time point can cause serious underestimation of the response, or even lead to misinterpretations, because drought affects different genes, to a different extent, and sometimes even in a different direction depending on the time of day.

Among the thousands of drought-responsive genes we identified, we were particularly interested in those that could potentially be involved in leaf growth regulation under drought. Genes with expression patterns matching to the dynamics of leaf growth under drought are enriched for genes involved in ethylene, JA, and GA biosynthesis and signaling. JA has previously been shown to be involved in the drought response, and some mutants in JA signaling (*coi1* and *jin1*) are known to have a less pronounced decrease in biomass upon exposure to long-term moderate drought (Harb *et al.* 2010). In contrast, ethylene and GA were previously described as central regulators of leaf growth inhibition of plants exposed to *in vitro* osmotic stress. Specifically in actively growing Arabidopsis leaves, ethylene accumulates and multiple genes encoding ethylene response factors (ERFs) are induced by short-term osmotic stress treatments, followed by a growth-regulatory cascade involving GA and the DELLA proteins as final regulators of the pathway to inhibit cell division and cell expansion (Claeys *et al.* 2012; Dubois *et al.* 2013; Skirycz *et al.* 2011a). Our data suggest that similar mechanisms might exist in developing leaves exposed to mild drought stress in soil. These findings were unexpected because ethylene and GA biosynthesis and signaling components were recently reported to be either not associated with the drought response, or to be underrepresented among the up-regulated genes, or enriched among the down-regulated genes (Baerenfaller *et al.* 2012; Clauw *et al.* 2015). Moreover, it was

suggested by Trontin *et al.* (2014) that previously identified stress-responsive ERFs may reflect a specific response to mannitol rather than a general osmotic stress response; here, we show that this is not the case. Among the genes specifically induced during the day but not during the night, the gene encoding ACC-synthase 8 (ACS8) is present. We hypothesize that the induction of ethylene triggers the activation of ERFs, such as *ERF2* and *ERF8*, which we showed to likely be involved in the early stress response to mild drought. Supporting this hypothesis, inhibition of ethylene biosynthesis in wheat has been shown to result in increased drought tolerance (Beltrano *et al.* 1999), and in maize, expression of a negative regulator of ethylene biosynthesis (ARGOS) or down-regulation of *ACS* genes enhances grain yield under drought (Habben *et al.* 2014; Shi *et al.* 2015). Finally, we also identified three genes encoding DELLA proteins (*GAI*, *RGL1* and *RGL2*) among the genes up-regulated in actively growing leaves under drought stress. As these genes were not identified in previous similar datasets obtained at later time points following onset of drought stress, we speculate that this induction of growth inhibitors might be specific to the early drought response, captured here in actively growing leaves. It is important to highlight that we explored only a small subset of potential regulators, and that growth is most likely fine-tuned by a robust and complex network of growth regulators to which many nodes contribute, as is reflected by the large number of genes that show small, yet significant, changes in expression in response to drought. Nonetheless, we can conclude that this unique approach combining high-resolution phenotyping and transcriptomics holds a huge potential to identify putative regulators underlying leaf growth inhibition under drought stress and that, surprisingly, these mechanisms might be similar to those previously observed under *in vitro* osmotic stress.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. **Experimental setup used to measure short-term response to mild drought**. (A) Arabidopsis plants were grown on the Weighing, Imaging and Watering Machine (WIWAM; www.wiwam.be). (B) Four seedlings were grown per pot to enable growth of a sufficient amount of young seedlings per experiment. Scale = 2 cm. (C) Leaf size was measured in a destructive way by harvesting the third leaf and measuring its size using a light microscope. Scale = 1 mm. (D) Leaf imprints in dental resin enable measurement of leaf size over time of the same leaf. Scale = 1 mm. (E) Scanning electron microscopy of nail polish replica of the red square of the imprints shown in (D). The selected zones contained about 200 epidermis cells. Growth of individual cells can be tracked over time as illustrated for two cells. Examples of new divisions are shown in the insets. Scale = 50 μm, DSLW = days since last watering.
Figure S2. **Time-course transcriptome analysis under drought**. (A) Overlaps between the datasets of drought-responsive genes. Values represent the amount of differentially expressed genes for drought vs. well-watered at each time point (FDR < 0.05). (B) Effect of mild drought on the amplitude of transcript oscillations. For the 6394 transcripts clearly oscillating under well-watered conditions, the amplitude of oscillation, defined as the relative difference in transcript level between the lowest and the highest expression level throughout a day, was

calculated under well-watered and mild drought conditions. Transcript oscillations were either not affected (gray), decreased (red), or increased (green) by drought. Drought also induced oscillations of transcripts that were not oscillating under well-watered conditions ("New").

Figure S3. **Genes affected by drought in opposite direction during day and night**. (A) Clustering of the differentially expressed genes from the RNAseq dataset based on their Log2(fold change) under drought yielded four clusters of genes which were significantly affected in the opposite direction by drought stress depending on the time of day. Among the 49 genes within the third cluster, multiple *KCS* genes encoding enzymes for very long chain fatty acid elongation were found. (B) Expression analysis of *DREB2A* during the four days following drought onset. Error bars indicate standard errors. DSLW = days since last watering, WW = well-watered.

Figure S4. **Expression analysis of core circadian clock genes under mild drought**. Expression of the core circadian clock regulators *LHY*, *TOC1* and *CCA1* at different times of day during four days following drought onset. PTreatment and PTreatment*Time of day represent P-values for the effect of drought and the interaction between drought and time of day, respectively (ANOVA; Treatment and Time of day as factors). DSLW = days since last watering, WW = well-watered, FC = fold change, Dr = drought, * $P < 0.05$ (ANOVA; Tukey HSD).

Figure S5. **Phenotypical analysis of *toc1* mutants**. Relative growth rate of wild type and *toc1* mutants during day and night under well-watered (WW) and drought conditions around the moment of growth inhibition (3 days since last watering).

Figure S6. **Confirmation by qPCR of putative regulators of leaf growth under drought stress**. Transcription factors selected from the list of Dataset S2 containing genes correlated (A) or anti-correlated (B) with the dynamics of leaf growth under drought stress. qPCR was performed on two additional biological repeats of the 8 PM and 4 AM' time points. The expression was considered as validated (genes in bold) when per time point the up- or down-regulation could be reproduced and when the tendency between the two time points was reproducible. FC = fold change, * = mutant used for growth analysis (see also Fig. S7).

Figure S7. **Phenotypic screen for candidate growth regulators under drought**. (A) Average area of the third leaf of mutants under well-watered (WW) or drought conditions measured at six days since last watering (DSLW), relative to the respective wild type under WW conditions. (B) Relative reduction in average leaf area caused by drought in each line at six DSLW. For both panels, error bars represent standard errors of two biological replicates.

Table S1. **Comparison of this study with other relevant datasets**. (A) Five publicly available datasets were relevant for comparison with this study, as they were performed on shoot tissue of plants exposed to mild or moderate drought stress. Severe and desiccation stress studies were excluded. All raw datasets were reanalyzed similarly as the dataset of this study, yielding the indicated number of differentially expressed genes based on significance (FDR < 0.05). Genes with very low fold changes (Log2FC < |0.2|) were also excluded. Upon reanalysis of the natural variants datasets (Clauw *et al*. 2015 and Des Marais *et al*. 2012) only 8 and 3 genes, respectively, were differentially expressed when using only the data for Col-0; therefore, the originally published list of differentially expressed genes (based on all accessions) was used for further comparison. (B) Overview of the 29 genes differentially expressed at all time points profiled in this study and comparison with the datasets presented in (A). Indicated values are the Log2(fold change) between drought and well-watered conditions at each time points. Colored cells are significantly different (FDR < 0.05).

Dataset S1. **Overview of the 5659 genes differentially expressed by drought in this dataset**. See *Materials and Methods* for details about the analysis of differential expression. Indicated values represent Log_2(fold change) of drought vs. well-watered conditions at each time point. Genes were considered specific for this dataset when they were not differentially expressed by drought in the datasets of Baerenfaller *et al*. (2012), Clauw *et al*. (2015), Harb *et al*. (2010), Wilkins *et al*. (2010) and Des Marais *et al*. (2012) upon reanalysis of the raw data in the same way as our data (see Table S1A for details about this dataset).

Dataset S2. **Overview of the 228 genes putatively involved in leaf growth regulation under drought**. The genes belong to clusters selected based on their expression pattern, correlating or anti-correlating with the dynamics of leaf growth under drought. Indicated values are Log_2(fold change) in drought vs. well-watered conditions. Colored cells are significantly differentially expressed (FDR < 0.05).

